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Transcriptional Regulation of ROS Controls Transition from Proliferation to Differentiation in the Root

Hironaka Tsukagoshi,[1] Wolfgang Busch,[1] and Philip N. Benfey[1,*]
[1]Department of Biology and Institute for Genome Science and Policy Center for Systems Biology, Duke University, Durham, NC 27708, USA
*Correspondence: philip.benfey@duke.edu
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SUMMARY

The balance between cellular proliferation and differentiation is a key aspect of development in multicellular organisms. Using high-resolution expression data from the *Arabidopsis* root, we identified a transcription factor, UPBEAT1 (UPB1), that regulates this balance. Genomewide expression profiling coupled with ChIP-chip analysis revealed that UPB1 directly regulates the expression of a set of peroxidases that modulate the balance of reactive oxygen species (ROS) between the zones of cell proliferation and the zone of cell elongation where differentiation begins. Disruption of UPB1 activity alters this ROS balance, leading to a delay in the onset of differentiation. Modulation of either ROS balance or peroxidase activity through chemical reagents affects the onset of differentiation in a manner consistent with the postulated UPB1 function. This pathway functions independently of auxin and cytokinin plant hormonal signaling. Comparison to ROS-regulated growth control in animals suggests that a similar mechanism is used in plants and animals.

INTRODUCTION

Growth in multicellular organisms depends on maintaining the proper balance between cell division and differentiation. Disruption of this balance in animals can lead to disease states such as cancer. In plants, because organs are continuously formed from stem cells, disruption of this balance leads to premature cessation of growth or abnormal organogenesis. In the root of *Arabidopsis*, cells originate from a stem cell center at the tip. Progeny of these stem cells rapidly divide in a transit-amplifying zone know as the meristem, after which they undergo massive increases in cell volume in the elongation zone. The transition from cellular proliferation to elongation marks the initial stage of differentiation and occurs at a slightly different point for each cell type, producing a somewhat jagged boundary of this transition zone (TZ). Once fully elongated, cells enter the maturation zone in which they differentiate into various cell types. Previous work has shown that root growth is determined by the rate of cell division in the meristematic zone and the extent of cell expansion in the elongation zone (Beemster and Baskin, 1998). Acceleration of root growth in *Arabidopsis* is correlated with increasing root meristem size as a result of increased rates of cell division and the delay of the onset of cell expansion (Ubeda-Tomas et al., 2009).

Several factors have been identified that are involved in the regulation of the transition from cellular proliferation to differentiation. The PLETHORA (PLT) proteins have been shown to determine the position of the stem cell niche and are required to maintain stem cell activity (Aida et al., 2004). There is also evidence that the balance between the hormones cytokinin and auxin in the TZ plays an important role in defining the size of the meristem (Dello Ioio et al., 2008). External applications of these hormones can change the position of the TZ. The underlying mechanism involves a cytokinin-dependent transcription factor, ARR1, which regulates the expression of another transcription factor, SHY2, which acts in auxin signaling (Dello Ioio et al., 2008). It is unclear whether this hormonal signaling interaction is the only means of controlling the transition from proliferation to differentiation.

Evidence for another pathway acting to maintain meristem function came from studies of the *ROOT MERISTEMLESS1* (*RML1*) gene. Mutations in this gene result in plants that are not able to establish an active root meristem. *RML1* encodes a glutathione biosynthetic enzyme, which has been shown to be important for regulating cellular redox states. Furthermore, it was shown that the G_1-to-S cell-cycle transition in synchronized tobacco cell suspension culture required an adequate level of glutathione (Vernoux et al., 2000). These results suggested that redox regulation plays an important role in maintaining root meristem activity. Moreover, this is supported by the finding that differences in superoxide and hydrogen peroxide accumulation in the root tip significantly affect root growth and differentiation (Dunand et al., 2007). In animals, there are numerous studies indicating that reactive oxygen species (ROS) distribution plays an important role in regulating cell state decisions (Owusu-Ansah and Banerjee, 2009; Sarsour et al., 2008). However, no molecular link between ROS distribution and cell status has been previously established at the transcriptional level in either plants or animals.

Here, we show that a bHLH transcription factor, UPBEAT1 (UPB1), modulates the balance between cell proliferation and differentiation by directly regulating the expression of a set of

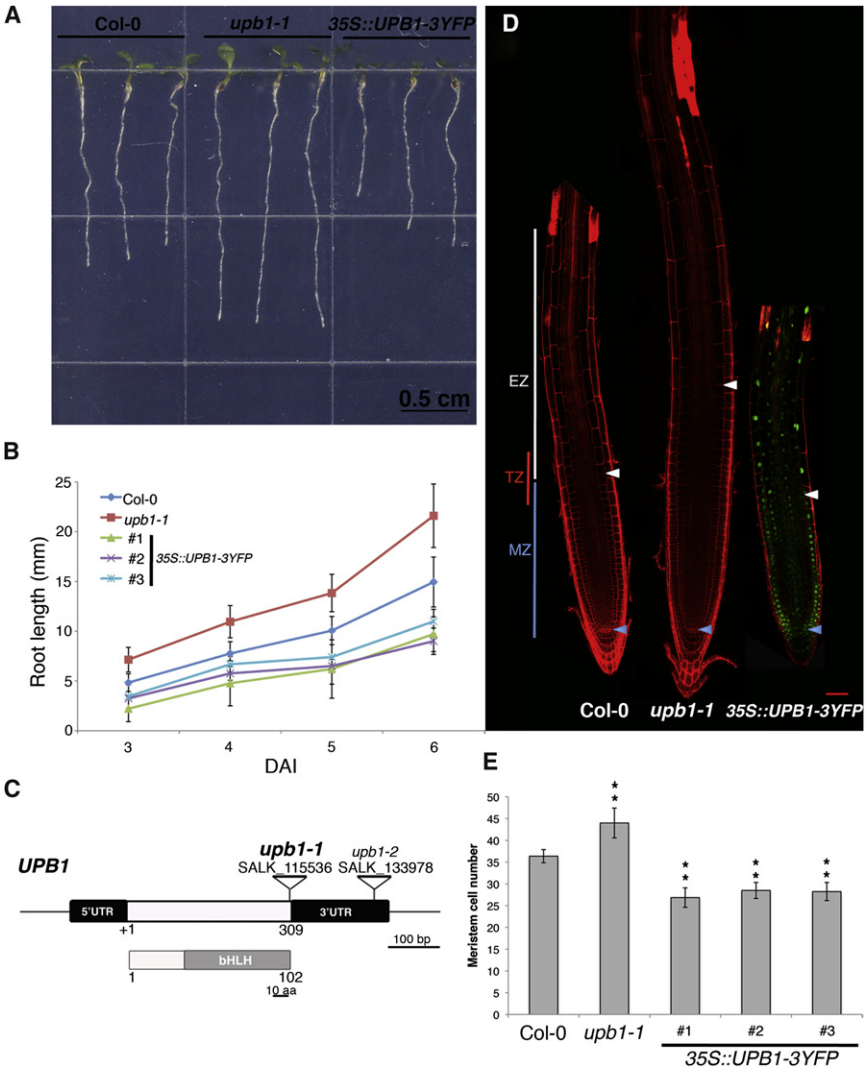

Figure 1. Features of the *upbeat1* (*upb1*) Mutant

(A) Wild-type Col-0, *upb1-1*, and *35S::UPB1-3YFP* seedlings 5 days after imbibition (dai).

(B) Average root length (*y* axis) of 50 individuals of Col-0 (blue), *upb1-1* (red), and 3 independent *35S::UPB1-3YFP* (green, purple, and light blue) lines. Measurements were taken over several days (*x* axis). Error bars depict standard deviation (SD).

(C) Genomic structure of *At2g47270* and position of the T-DNA insertion of *upb1-1* and *upb1-2* mutants. The predicted protein is depicted below. Gray shaded box shows basic helix-loop-helix domain (bHLH).

(D) Root tip morphology of 6 dai Col-0, *upb1-1*, and *35S::UPB1-3YFP* (line #2) plants. Blue arrowheads mark quiescent center (QC) cells, whereas white arrowheads indicate cortex transition zones. Scale bar, 50 μm.

(E) Average number of cells in the root meristems (*y* axis) of Col-0, *upb1-1*, and 3 independent *35S::UPB1-3YFP* lines at 6 dai ($n > 50$, ± SD); ** $p < 0.001$, as determined by a Student's t test. See also Figure S1.

peroxidases. Peroxidases are known to regulate the levels of certain ROS, particularly hydrogen peroxide and superoxide. Staining for the presence of these ROS in the root and altering their concentrations using chemical reagents showed a clear correlation between growth rate, location of the TZ, and the relative distribution of different ROS species in the meristematic and elongation zones. Interestingly, differences in the localization of UPB1 in transcriptional and translational reporter lines suggest that this transcriptional regulator might also function as an intercellular signaling molecule. We show evidence that UPB1 provides a direct transcriptional link between ROS distribution and the proliferation status of the cells in the root tip.

RESULTS

UPBEAT1 Controls the Transition from Cellular Proliferation to Differentiation

To identify transcription factors (TFs) that regulate the first stages of the transition from cellular proliferation to differentiation, we analyzed the RootMap gene expression data (Brady et al., 2007a) and identified approximately 100 TFs with increased expression at the boundary between the meristematic and elongation zone, which marks the onset of differentiation. We screened T-DNA insertional mutant lines available for 96 of these genes, looking for alterations in primary root growth rates. One line (SALK_115536) developed a longer root than wild-type and contained an insertion in *At2g47270* (Figures 1A and 1B). This gene showed a particularly prominent expression peak at the boundary between the meristematic and elongation zone (Figure S1A available online). By qRT-PCR, we confirmed that the T-DNA insertion caused a strong reduction in expression of *At2g47270*, which encodes an uncharacterized protein with a bHLH domain (Figures S1B and S1C). The bHLH domain occupies 70% of the 102–amino acid protein, which we named UPBEAT1 (UPB1) (Figure 1C). The *Arabidopsis* genome contains 147 genes that are predicted to encode proteins with a bHLH domain. UPB1 belongs to the bHLH-subfamily 14, but it is only distantly related to the other members of that subfamily (Toledo-Ortiz et al., 2003). Apart from the bHLH domain, no other functional domains were predicted in UPB1.

To determine the effects of the insertional mutation (*upb1-1*) on root growth, we counted the number of cortex cells in a cell file extending from the quiescent center (QC) to the first elongated cell, as a measurement of meristem size (Dello Ioio et al., 2007). We found a significant increase in cortex cell number in the *upb1-1* mutant, indicating enlargement of the meristem (Figures 1D and 1E). No difference was detected in

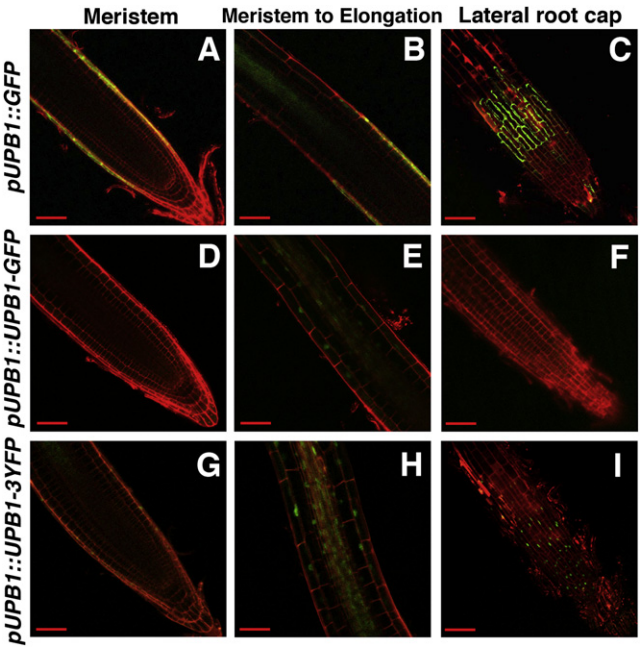

Figure 2. *UPB1* Gene and Protein Expression Patterns in the Root
(A–C) Expression of *pUPB1::GFP* (transcriptional fusion) in wild-type.
(D–F) Localization of *pUPB1::UPB1-GFP* (translational fusion) in *upb1-1* background.
(G–I) Localization of *pUPB1::UPB1-3YFP* in *upb1-1* background.
The three zones with GFP signal are depicted: meristematic zone (A, D, and G), transition zone (B, E, and H), and the lateral root cap (C, F, and I). Note that the lateral root cap images are of the surface of the root, whereas the meristematic and transition zone images are of median longitudinal optical sections. Scale bars, 50 μm. See also Figure S2.

the radial pattern of root cell layers between *upb1-1* and wild-type, suggesting that *UPB1* functions primarily in regulating root growth as opposed to patterning. The only other available T-DNA insertion was located at the end of the 3′ UTR of *UPB1*. We were unable to detect any phenotypic effects or significant UPB1 expression level changes in this line. To further explore the function of UPB1, we created ectopic expression lines with the constitutive 35S promoter and a fluorescent reporter (*35S::UPB1-3YFP*). Three independent lines showed reduced root length and a decrease in cortex cell number in the meristematic zone. (Figure 1). To determine whether cell size was also regulated by UPB1, we measured the length of the first cell in the maturation zone in *upb1-1* and in the *35S::UPB1-3YFP* transformants. In *upb1-1*, cells were longer than in wild-type, whereas in the *35S::UPB1-3YFP* lines, cells were shorter than in wild-type (Figure S1D). Taken together, these results suggest that UPB1 acts as a regulator of root growth through modulation of the transition from cell proliferation to elongation as well as playing a role in controlling cell size.

The UPB1 Protein Appears to Move from the Lateral Root Cap to the Elongation Zone

A correlation exists between the position of the lateral root cap (LRC) furthest from the root tip and the TZ (Willemsen et al., 2008), but the underlying mechanism is unknown. The tissue-specific expression data indicate that *UPB1* mRNA is expressed in the vascular tissue as well as in the COBRA-like 9 (COBL9) expression domain, which includes root hair and LRC (Brady et al., 2007a, 2007b). To begin to determine whether UPB1 might play a role in the signaling process between the LRC and the TZ, we constructed transcriptional and translational fusions and transformed them into plants. For the transcriptional reporter, we used 3002 bp upstream of the putative start codon fused to GFP (*pUPB1*::GFP). Five independent lines exhibited strong fluorescence in cells of the LRC close to the TZ. Outside of the LRC, fluorescence was detected in the vascular tissue of the elongation and maturation zones (Figures 2A–2C). For the translational reporter, we used the same promoter upstream of the UPB1-coding region fused to GFP (*pUPB1::UPB1-GFP*). Interestingly, in plants containing this construct, we detected low GFP fluorescence in the LRC and the meristematic zone. Fluorescence was primarily localized to the nuclei of all cell types in the elongation zone (Figures 2D–2F). We also detected weak fluorescence in the maturation zone. Expression of the translational fusion in the *upb1-1* line rescued the mutant phenotype (Figures S2A–S2C), indicating that the fusion protein functions in a manner similar to the native UPB1 protein. The difference between mRNA and protein localization suggests that the UPB1 protein might move from the LRC or vascular tissue to function in all cell files in the elongation zone. To test this, we used the same promoter but fused the *UPB1*-coding region to a triple yellow fluorescent protein (3YFP) tag, because this tag has a high molecular weight and has been used to reduce protein movement (Kurata et al., 2005). In *pUPB1::UPB1-3YFP* plants, YFP fluorescence was detected primarily in the nucleus of the LRC, in addition to all cells in the elongation zone (Figures 2G–2I). We conclude from this localization pattern that movement was reduced in these lines but not eliminated. Consistent with this observation, the mutant phenotype was partially rescued (Figure S2D).

Identification of Genes Regulated by UPB1

The spatial distribution of the UPB1 protein suggested that it might exert a different effect on gene expression in the meristematic and elongation zones. Therefore, we isolated the meristematic and elongation zones by microdissection and extracted RNA from each section independently. In the meristematic zone of *upb1-1* mutants, expression of only 55 genes was significantly altered in comparison to wild-type (2-fold change, $p < 0.05$), whereas expression of 738 genes was affected in the elongation zone of the mutant roots (Figure 3A). This finding was consistent with the hypothesis that UPB1 is primarily active in the elongation zone. Further support came from microarray analysis of ectopic expression of *UPB1* in the *upb1-1* mutant. Stronger transcriptional effects were observed in the meristematic zone (1809 genes) than in the elongation zone (812 genes). If UPB1 normally acts in the elongation zone, then ectopic expression in the meristematic zone would be expected to have a greater effect there than in the elongation zone where it normally functions. Because of the minimal effects of the *upb1-1* mutation on gene expression in the meristematic zone, we did not include these data in our subsequent analyses.

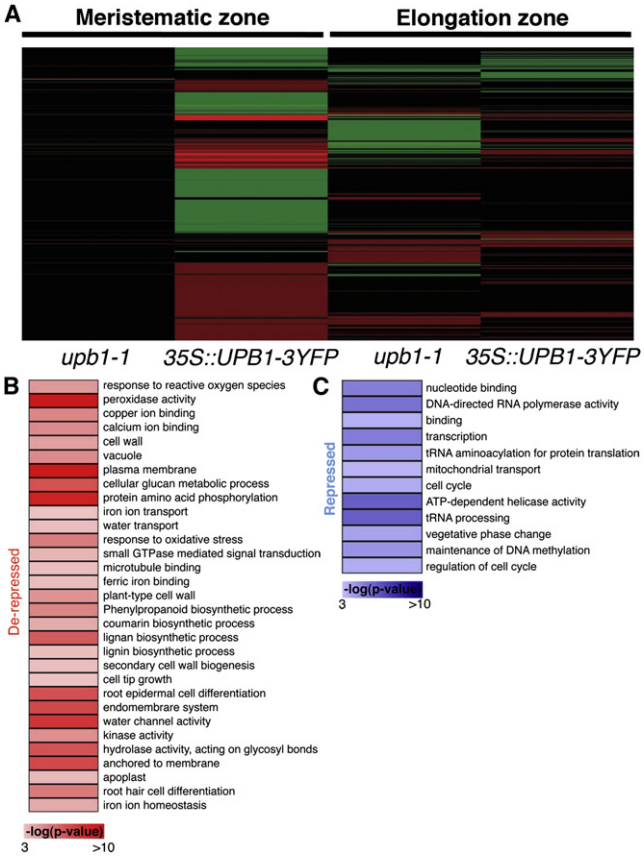

Figure 3. Expression and Function of *UPB1* Response Genes

(A) Transcriptome changes upon alteration of *UPB1* expression levels. The heat map includes all genes that were differentially expressed in at least one of the microarray experiments. Green indicates an increase in expression, red indicates a decrease in expression; color intensity indicates the magnitude of the effect.

(B and C) Enriched Gene Ontology (GO) categories within gene lists consisting of genes that are negatively (B) or positively (C) regulated by UPB1.

See also Figure S3 and Table S1.

Meta-analysis approaches to integrate diverse and inherently noisy data have proven useful to identify transcription factor target genes (Busch et al., 2010; Levesque et al., 2006). We performed a meta-analysis of the three datasets in which significant changes occurred. Analogous to Busch et al. (2010), we obtained a cumulative Z score (cZS) for each gene and empirically determined a significance threshold for the cZS by random sampling (see Supplemental Information for details). Using the significance threshold of $p < 0.01$, we identified 2375 UPB1-responsive genes that exhibited consistent expression changes upon modulation of *UPB1* expression (Table S1). To investigate the biological function of these genes, we identified significantly enriched Gene Ontology (GO) categories (Figures 3B and 3C). GO enrichment analysis associates each gene of a list with distinct biological processes and then evaluates whether the list contains more genes than expected by chance for a certain biological process. The most significantly enriched GO category of genes negatively regulated by UPB1 was "peroxidase activity" ($p < 10^{-11}$). Another highly enriched category was "response to reactive oxygen species" ($p < 10^{-4}$). We found these to be particularly interesting because recent reports indicated that peroxidases and ROS play an important role in controlling root growth (Dunand et al., 2007; Passardi et al., 2006). The ROS produced by peroxidases is essential in the peroxidase-mediated formation of lignin (Ros Barcelo, 1997), which is required for the formation of primary cell walls. We also found that "lignin biosynthetic process" ($p < 10^{-3}$) and "lignan biosynthetic process" ($p < 10^{-6}$) GO categories were overrepresented within derepressed genes in *upb1-1*. Further indication that UPB1 regulates cell wall remodeling is found in other enriched GO categories: "cellular glucan metabolic pathway" ($p < 10^{-6}$), "cell wall" ($p < 10^{-4}$), "phenylpropanoid biosynthesis" ($p < 10^{-5}$), and "secondary cell wall biogenesis" ($p < 10^{-3}$). These findings raise the possibility that UPB1 controls growth by regulating ROS activity, which in turn regulates cell wall remodeling.

Among genes that were positively regulated by UPB1, the enriched GO categories were "tRNA processing" ($p < 10^{-6}$), "ATP-dependent helicase activity" ($p < 10^{-6}$), "DNA-directed RNA polymerase activity" ($p < 10^{-5}$), "maintenance of DNA methylation" ($p < 10^{-4}$), and "regulation of cell cycle" ($p < 10^{-3}$). These would also appear to be involved in growth and replication processes although we think it is likely that these are indirect responses. In particular, the genes in the cell cycle GO category contained both S-phase and M-phase activated genes, indicating that there is no specificity for a particular phase of the cell cycle. We note that we did not find enrichment for genes involved in plant hormone homeostasis or signaling.

Identification of UPB1 Direct Targets

To identify UPB1 direct targets, we performed chromatin immunoprecipitation of UPB1 followed by hybridization to a custom oligonucleotide microarray (ChIP-chip). We reasoned that a subset of the genes found by microarray expression analysis should be overrepresented in the ChIP-chip binding data. On the basis of this assumption, we systematically explored the parameter space to optimize our detection settings for enriched regions in the ChIP-chip experiments (for details, see Extended Experimental Procedures). By using highly stringent conditions in which transcriptionally regulated genes were enriched, we identified 166 putative UPB1 direct target genes (Table S2). These included genes of various annotated functions, including quite a few transcription factors indicating that UPB1 might regulate a cascade of transcription factors. Of particular interest were three peroxidase genes (Figure 4A) that are expressed highly at the boundary of the meristematic and elongation zone in the RootMap datasets (Figure S3). All three (*At4g11290*; *Per39*, *At4g16270*; *Per40*, *At5g17820*; *Per57*) are up-regulated in the *upb1-1* mutant and down-regulated in the UPB1 ectopic expressor (Figure 4B). Consistent with these results, the phenotype of an overexpressor of another peroxidase gene (*Per34*) had longer roots than wild-type, whereas the double knockdown line of *Per33* and *Per34* had shorter roots (Passardi et al., 2006). To investigate whether the UPB1 target peroxidases also play a role in root growth, we ectopically expressed *Per57* by driving its cDNA with the *35S* promoter. Three independent lines

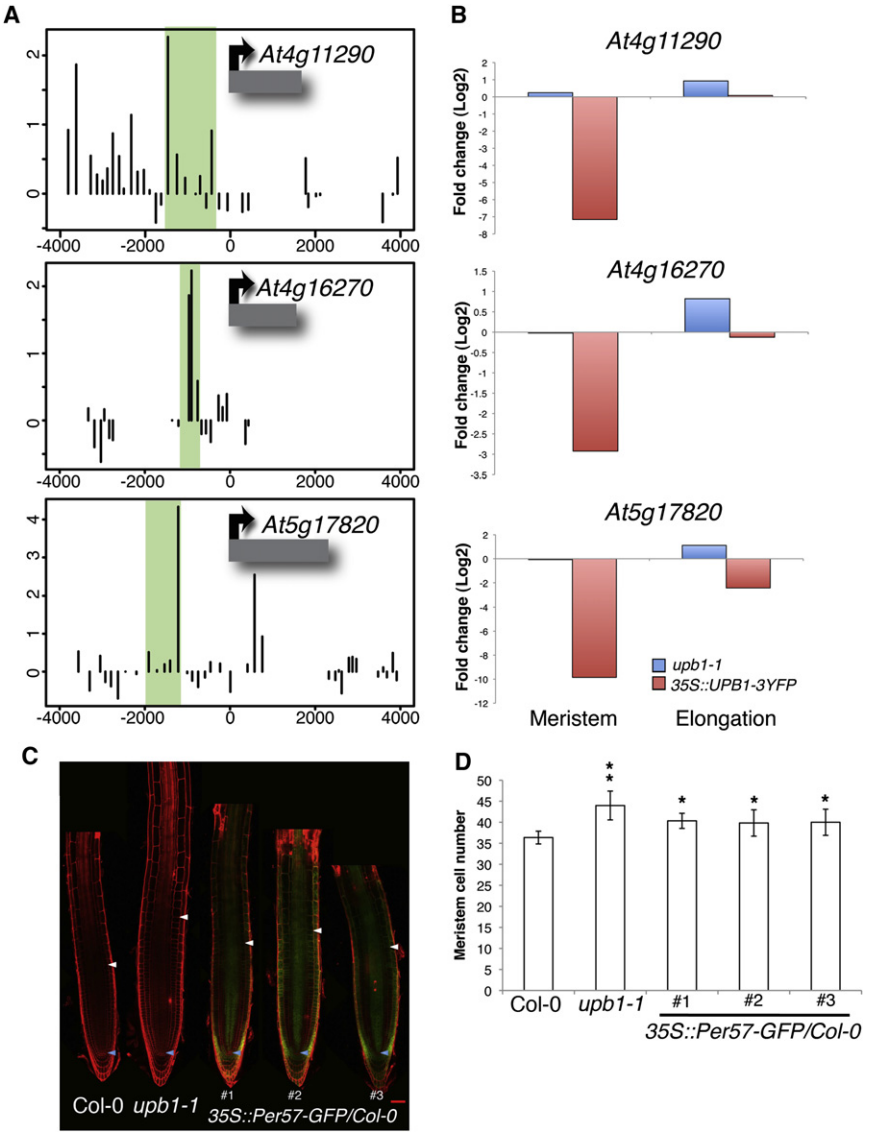

Figure 4. UPB1 Binding of Upstream Regulatory Regions and Resulting Transcriptional Effects

(A) Binding profile of UPB1 to upstream regulatory regions of *At4g11290*, *At4g16270*, and *At5g17820*. UPB1-bound chromosomal regions are shown by average Z scores of enrichments in the 4 ChIP-chip experiments (*y* axis). Genomic positions (*x* axis) are given relative to the annotated transcription start of the indicated primary RNA. Shaded areas indicate genomic regions that were detected as enriched.

(B) Expression profiles of UPB1 direct targets, *At4g11290*, *At4g16270*, and *At5g17820* from microarray data.

(C) Root tip morphology of 6 dai Col-0, *upb1-1*, and *35S::Per57-GFP* (line #1 to #3) plants. Blue arrowheads mark QC cells; white arrowheads indicate cortex transition zones. Scale bar, 50 μm.

(D) Average number of cells in root meristems of Col-0, *upb1-1* and 3 independent lines of *35S::Per57-GFP* (line #1 to #3) plants ($n > 20$, ± SD; $^{**}p < 0.001$, Student's t test; $^{*}p < 0.05$).

See also Table S2 and Figure S7.

displayed a significantly larger meristem than wild-type. The phenotype was not as pronounced as in *upb1-1* mutants (Figures 4C and 4D), suggesting that the peroxidases directly controlled by UPB1 play additive roles in regulating root growth.

Redox Processes Are Important for Regulating Root Growth

Upon treatment with H_2O_2 for 24 hr, the root meristem in wild-type became significantly shorter (Figures 5A and 5C) and root length was reduced. In contrast, H_2O_2 treatment of the *upb1-1* mutant and UPB1 ectopic expressor did not result in a significant change of meristem size (Figures 5A, 5F, and 5I) or growth. Scavenging H_2O_2 by treating with potassium iodide (KI) in wild-type resulted in a longer root, which contained a larger root meristem (Figures 5A and 5D). The same treatment had no effect on the meristem size in the *upb1-1* mutant but significantly increased the meristem size of the *UPB1* ectopic expressor (Figures 5A, 5G, and 5J). The insensitivity of the *upb1-1* mutant to H_2O_2 scavenging is consistent with the up-regulation of peroxidase genes and a decrease in H_2O_2 levels (Figure S4). Moreover, subjecting the mutant to additional H_2O_2 has no effect on meristem size (Figure 5), consistent with the idea that the upregulated peroxidases are able to continually scavenge even an excess of H_2O_2.

To directly investigate the role of peroxidases in root growth, we treated plants with salicylhydroxamic acid (SHAM), an inhibitor of peroxidase activity (Brouwer et al., 1986). Wild-type meristems treated with SHAM became significantly smaller than those of untreated roots. The same treatment led to a decreased size of the meristem in *upb1-1* plants, to the extent that they were similar to untreated wild-type roots (Figure 5K). Conversely, the inhibitor had almost no effect on root meristem size in the *UPB1* ectopic expressor (Figure 5K).

Using a different peroxidase inhibitor, KCN (Bestwick et al., 1997), which inhibits a broader spectrum of peroxidases (Chen and Asada, 1989), even stronger effects could be observed. These included a decrease of meristem size in the *UPB1* ectopic expressor (Figure 5K).

These results indicated that H_2O_2 content and the regulation of H_2O_2 by UPB1-controlled peroxidase is important for root growth. To investigate the spatial aspect of this regulation, we determined the distribution of H_2O_2 by applying 3′-(p-hydroxyphenyl) fluorescein (HPF) to roots. HPF is known to stain

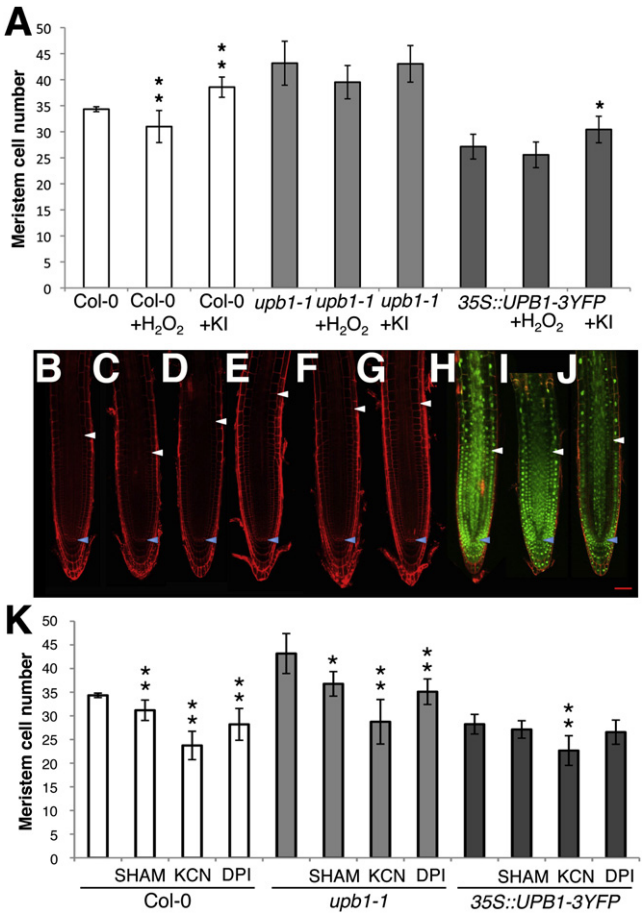

Figure 5. Effects of ROS Level Changes

(A) Average number of cells in root meristems of Col-0, *upb1-1*, and *35S::UPB1-3YFP* #2 plants (n > 30, ± SD; **p < 0.001, Student's t test; *p < 0.05).

(B–J) Root meristems of 6 dai plants upon various treatments for 24 hr. Scale bars, 50 μm. Panels show untreated Col-0 plant (B), 100 μM hydrogen peroxide (H_2O_2) (C), 1 mM potassium iodide (KI) treated Col-0 plants (D), untreated *upb1-1* mutant (E), 100 μM H_2O_2 (F), 1 mM KI treated *upb1-1* mutants (G), untreated *35S::UPB1-3YFP* #2 (H), 100 μM H_2O_2 (I), and 1 mM KI treated *35S::UPB1-3YFP* #2 plants (J); blue arrowheads indicate cells of the QC and white arrowheads indicate the cortex transition zone.

(K) Average number of cells in root meristems of Col-0, *upb1-1* and *35S::UPB1-3YFP* plants after 24 hr treatment with 100 μM SHAM, 100 μM KCN, and 100 μM DPI (n > 30, ± SD; **p < 0.001, Student's t test; *p < 0.05). See also Figure S4.

hydrogen peroxide (Dunand et al., 2007). The intensity of HPF fluorescence changed according to our expectations after different treatments that modified ROS levels in roots (Figures S4A–S4M). In wild-type, strong HPF fluorescence was detected in the columella, the LRC, in the vasculature, and the epidermis of the elongation zone. Fluorescence was substantially weaker in the meristematic zone than in the elongation zone (Figures 6B and 6D). In the *upb1-1* mutant, less HPF fluorescence in the entire root was observed (Figures 6A and 6D). Conversely, in the *UPB1* ectopic expressor, HPF fluorescence was increased (Figures 6C and 6D). We also used the highly specific H_2O_2 indicator, BES-H_2O_2-Ac (Maeda, 2008; Maeda et al., 2004), with results similar to those of HPF staining (Figures 6E–6H). These two independent assays provide strong support for our hypothesis that increased peroxidase activity in *upb1-1* results in lower H_2O_2 levels, whereas repression of peroxidases in the meristem in the ectopic expression lines results in an increase in H_2O_2 levels.

Another aspect of ROS growth regulation involves superoxide ($O_2^{\bullet -}$), which is thought to be produced by the activity of NADPH oxidases and has been shown to affect root growth and root hair development (Foreman et al., 2003). To investigate the role of $O_2^{\bullet -}$ in UPB1-regulated root growth, we treated plants with diphenylene iodonium (DPI), which primarily inhibits NADPH oxidase activities. In both wild-type and *upb1-1*, treatment with DPI resulted in a reduction in meristem size but there was no effect on the meristem of the UPB1 ectopic expressor (Figure 5K). Furthermore, to determine the distribution of $O_2^{\bullet -}$ in the root, we stained roots with nitroblue tetrazolium (NBT), which is widely used as an indicator of $O_2^{\bullet -}$ levels (Bielski et al., 1980). In wild-type, strong staining appeared in all cell types in the meristematic zone, whereas only the vascular tissue in the elongation zone showed staining, indicating that $O_2^{\bullet -}$ preferentially accumulates in the root meristematic zone (Figure 6J). In *upb1-1*, staining of the meristematic and elongation zones was more intense compared to wild-type plants (Figures 6I and 6L). Interestingly, the $O_2^{\bullet -}$ level in wild-type roots treated with KI appeared similar to that of *upb1-1* roots (Figures S4N–S4Z). In contrast, in the *UPB1* ectopic expressor, staining of the meristematic and elongation zones appeared weaker than in wild-type (Figures 6K and 6L). We also used dihydroethidium (DHE) as a second indicator for $O_2^{\bullet -}$ (Owusu-Ansah et al., 2008). DHE fluorescence was similar to the NBT staining (Figures 6M–6P). These results are consistent with UPB1 functioning as a regulator of ROS production through repression of peroxidase gene expression. Finally, we performed simultaneous staining for H_2O_2 and $O_2^{\bullet -}$ by using BES-H_2O_2-Ac and DHE on the same roots (Figures 6Q–6S). The simultaneous staining results suggest that there are opposing gradients of H_2O_2 and $O_2^{\bullet -}$. The crossing point of these gradients is altered in plants with modified UPB1 expression and coincides in each case with the onset of differentiation. This raises the possibility that the crossing point of the two gradients might determine the position of the TZ in the *Arabidopsis* root tip.

To determine whether ROS also influences the process of cell expansion, we measured the size of the first mature cells in *upb1-1*, the *UPB1* ectopic expressor and the *Per57* overexpressor, as well as after different ROS treatments. We found a positive correlation between cell length and meristem size (Figure S5C).

Finally, we examined *UPB1* expression after treatment with H_2O_2 and KI. H_2O_2 treatment caused up-regulation of *UPB1* expression, whereas KI treatment reduced *UPB1* expression (Figure S5A). This indicates that H_2O_2 levels regulate UPB1 expression creating a feed back loop in ROS signaling.

Taken together, our results are consistent with a model in which UPB1 acts to repress peroxidase expression in the elongation zone. In the *upb1-1* mutant, this results in an increase in $O_2^{\bullet -}$ and a decrease in H_2O_2 in this region of the root (Figure S5B). The balance of these ROS molecules appears to be

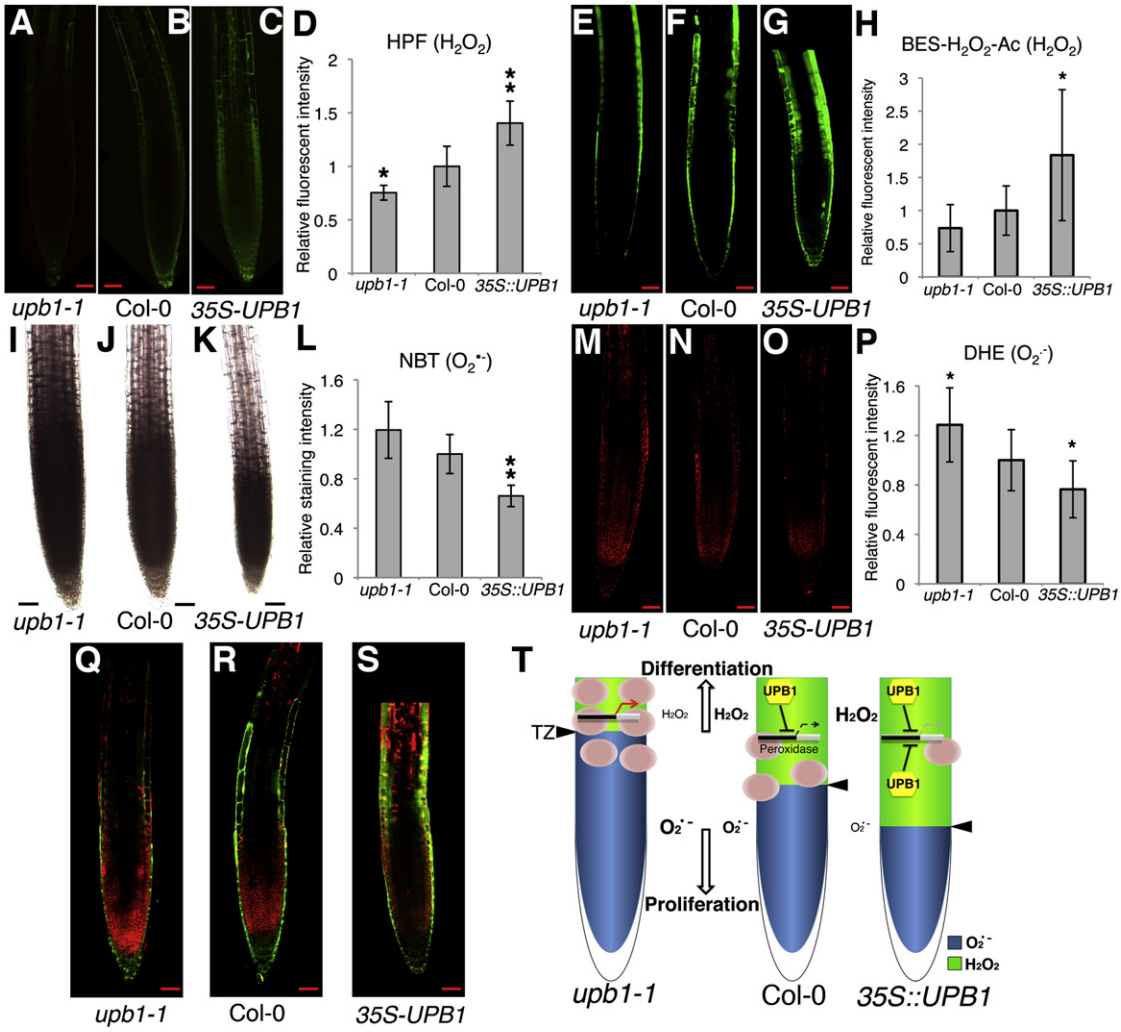

Figure 6. ROS Distribution and Proliferation/Differentiation

(A–S) Six dai roots stained with 3′-(p-hydroxyphenyl) fluorescein (HPF) (A–C), BES-H_2O_2-Ac (E–G), nitroblue tetrazolium (NBT) (I–K), dihydroethidium (DHE) (M–O), and both DHE and BES-H_2O_2-Ac (Q–S). Panels show *upb1-1* (A, E, I, M, and Q), Col-0 (B, F, J, N, and R), *35S::UPB1-3YFP #2* (C, G, K, O, and S). Scale bars, 50 μm. Quantification of HPF (D), BES-H_2O_2-Ac (H), and DHE fluorescent intensity (P) are shown (n = 20, ± SD). Quantification of NBT staining intensity is seen (L) (n = 20, ± SD; **p < 0.001, Student's t test; *p < 0.05).

(T) Model of UPB1-dependent regulation of meristem size. In Col-0 root tips (center panel), superoxide ($O_2^{\bullet-}$) accumulates in the meristem (blue area), whereas hydrogen peroxide (H_2O_2) accumulates in the elongation zone (green area). UPB1 represses expression of peroxidases (pink circles) in the elongation zone. In the *upb1-1* mutant (left panel) peroxidases are derepressed and higher abundance of peroxidases leads to increased content of $O_2^{\bullet-}$. On the other hand, UPB1 ectopic expressor (right panel) represses peroxidases and leads to increased levels of H_2O_2.

See also Figure S5.

important for making the transition from cell proliferation to differentiation (Figure 6T).

UPB1 Does Not Appear to Act through Cytokinin and Auxin Signaling

Our data strongly implicate ROS as being central to UPB1 function in regulating root growth. It is known that the ratio of cytokinin to auxin is important in controlling the balance of cell division and differentiation in the root, and that two key transcription factors, SHY2/IAA3 and ARR1, control this process (Dello Ioio et al., 2008). The *arr1* mutant has a large meristem phenotype similar to that of *upb1-1*. A first indication that UPB1 is not involved in the same signaling pathway as *ARR1* and *SHY2* came from examination of our microarray datasets in which neither gene appears to be responsive to UPB1 (Table S1). We confirmed this by qRT-PCR (Figure S6).

Exogenous cytokinin application decreased the meristem size of *upb1-1* in a similar fashion to that of wild-type, whereas exogenous auxin increased the meristem cell number in *upb1-1* and in the *UPB1* ectopic expressor in a manner similar to wild-type (Figure 7). Furthermore, exogenous application of auxin and cytokinin had almost no effect on *UPB1* expression in either

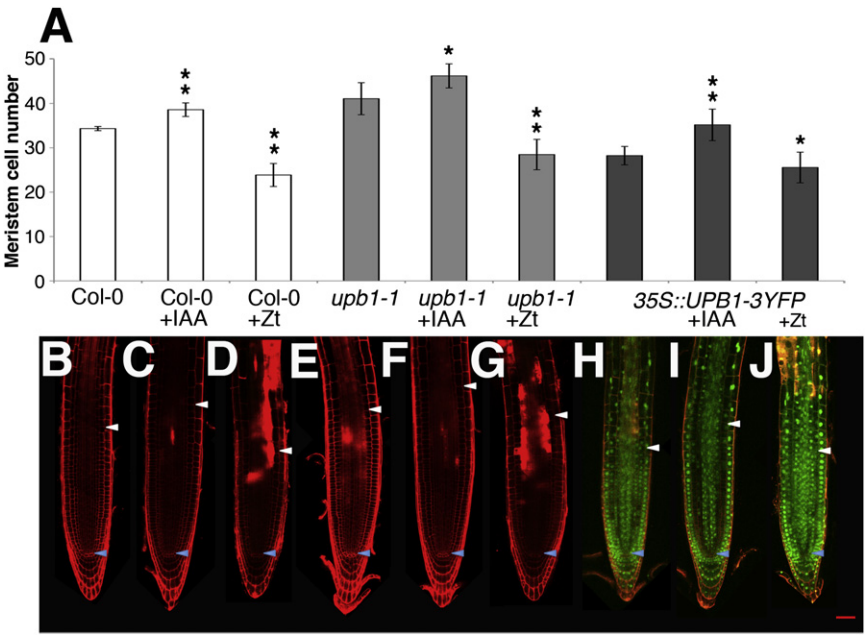

Figure 7. Effects of Cytokinin and Auxin on Developmental Zones of the Root

(A) Average number of cells in root meristems of Col-0, *upb1-1*, and *35S::UPB1-3YFP* #2 plants ($n > 30$, ± SD; $^{**}p < 0.001$, Student's t test; $^{*}p < 0.05$). (B–J) Root meristems of 6 dai plants after hormonal treatment for 24 hr (scale bars, 50 μm): untreated Col-0 plant (B), 0.5 nM IAA (C), 5 μM transzeatin (Zt) treated Col-0 plants (D), untreated *upb1-1* mutant (E), 0.5 nM IAA (F), 5 μM Zt treated *upb1-1* mutants (G), untreated *35S::UPB1-3YFP* #2 (H), 0.5 nM IAA (I), and 5 μM Zt treated *35S::UPB1-3YFP* #2 plants (J). Blue arrowheads indicate cells of the QC and white arrowheads indicate the cortex transition zone.

See also Figure S6.

the wild-type meristem or elongation zone (Figure S6). These results indicate that UPB1-mediated regulation of meristem size is likely to be independent of auxin and cytokinin signaling.

DISCUSSION

UPB1 Regulates ROS Signaling to Control the Transition from Proliferation to Differentiation

Our microarray expression analysis coupled with UPB1 ChIP-chip analysis indicated that UPB1 directly represses a set of peroxidases as cells begin to differentiate. The use of chemical inhibitors and the ectopic expression of one of the target peroxidase genes (*Per57*), as well as chemical indicators for ROS provided strong evidence that these peroxidases control ROS distribution, which in turn governs the transition from proliferation to differentiation. Further support for this hypothesis comes from treatment with peroxidase inhibitors, which cause a reduction in the size of the root meristem, indicating an earlier onset of differentiation (Figure 5K).

These genetic and chemical studies reveal the importance of peroxidase activity in the root tip and are consistent with our interpretation of the *upb1-1* phenotype as being directly related to UPB1 regulation of at least three Class III peroxidases. In *Arabidopsis*, there are 73 Class III peroxidase genes (Tognolli et al., 2002) of which 60 are represented on the ATH1 array, and 21 of these were affected by UPB1. All 21 genes are expressed primarily in the elongation zone (Figure S3). We obtained T-DNA insertion lines for many of the peroxidases that are UPB1 targets, but the single mutants did not show obvious phenotypes (data not shown), probably because of functional redundancy.

Peroxidases have two opposite functions: one is the reduction of H_2O_2 by moving electrons to various donor molecules and the second is to catalyze the hydroxylic cycle, which results in the formation of ROS, particularly $O_2^{\bullet -}$. In the *upb1-1* mutant, the distribution of $O_2^{\bullet -}$ and H_2O_2 was altered apparently as the result of derepression of a set of peroxidases. In living organisms, H_2O_2 is more stable than $O_2^{\bullet -}$ (Pitzschke et al., 2006). $O_2^{\bullet -}$ is transformed into H_2O_2 both spontaneously and through enzymatic activity of superoxide dismutase and other enzymes, such as apoplastic oxalate oxidase (Caliskan and Cuming, 1998), diamine oxidase (Federico and Angelini, 1986), or peroxidase (Elstner and Heupel, 1976). Our results indicate that, in wild-type, $O_2^{\bullet -}$ accumulates primarily in the meristematic zone whereas H_2O_2 accumulates mainly in the elongation zone. Given the changes in ROS distribution in *upb1-1* and *35S::UPB1-3YFP*, it would appear that $O_2^{\bullet -}$ and H_2O_2 distribution are important for localization of the transition zone. In the *upb-1* mutant, the consumption of H_2O_2 by peroxidases in the elongation zone might drive production of $O_2^{\bullet -}$ in the meristematic zone to maintain ROS homeostasis. In *35S::UPB1-3YFP*, these peroxidases are repressed, which leads to accumulation of H_2O_2 in the meristematic zone. Consistent with this interpretation, SHAM treated roots have H_2O_2 accumulation in the meristematic zone (Figure 6, Figure S4, and Dunand et al., 2007) and at least five NADPH oxidase genes were up-regulated in the *upb1-1* mutant (Table S1). We also determined the meristem size of 3 NADPH oxidase mutants (*AtrbohC*, *AtrbohD*, and *AtrbohC*). None of them showed a reduction in meristem size (data not shown). This finding suggests that there is functional redundancy among these genes for root meristem maintenance because inhibition of NADPH oxidase activity by DPI resulted in a reduction in meristem size (Figure 5K).

These results lead to the following working model. Maintenance of cellular proliferation requires an accumulation of $O_2^{\bullet -}$, whereas cellular differentiation requires elevated H_2O_2 levels. These two different ROS environments coincide with the meristematic and the elongation zone, as visible in the double staining of H_2O_2 and $O_2^{\bullet -}$. Because of the gradient nature of the ROS species distribution, the cells that enter the transition zone can still proliferate. Once the ratio between $O_2^{\bullet -}$ and H_2O_2 concentrations reaches a certain level, the cells stop

proliferating and begin to elongate (Figure 6T). According to this working model, the ROS balance in the transition zone plays a critical role and UPB1 is one of the key regulators in maintaining this balance. Because H_2O_2 itself affects *UPB1* expression, this regulatory system contains a feedback loop that might play a role in ROS homeostasis. Because high levels of ROS can damage cells, a feedback loop could allow the plant to maintain proper ROS levels. It would also constitute a system for adjusting ROS levels to maintain the proper balance between proliferation and differentiation.

To further investigate this hypothesis, we mined the oxidative stress data available in the public microarray databases. The AtGenExpress dataset contains experiments in which methyl viologen, a compound that causes continuous formation of $O_2^{\bullet -}$ (Asada, 2006) was used. *UPB1* was up-regulated after 24 hr of treatment, and the peroxidases that are direct targets of UPB1 showed decreased expression. Some peroxidases that are not targets of UPB1 showed increased expression, indicating that there might be compensatory expression responses to maintain ROS homeostasis (Figure S5D). Alternatively, the up-regulated peroxidases might be expressed in other organs or tissues.

Other TFs have been implicated in ROS signaling in the *Arabidopsis* shoot. In the *ascorbate peroxidase 1* (*apx1*) mutant, which is a cytosolic H_2O_2-scavenging enzyme in the *Arabidopsis* leaf, heat shock factor 21 (HSF21) was identified as a key transcriptional regulator for ROS response upon light stress (Davletova et al., 2005a). A dominant negative construct for HSF21 impaired ZAT12 expression, which was known as a transcriptional regulator of oxidative stress responses (Davletova et al., 2005b). However, neither HSF21 nor ZAT12 expression was affected in our microarray data. Furthermore, the meristem size of the *apx1* mutant was the same as wild-type (data not shown). This may indicate that different ROS sensing and signaling systems exist in different tissues or organs.

Redox homeostasis is also important for ROS signal transduction. Thioredoxins (TRX) are known as the key regulators for cell redox homeostasis (Meyer et al., 2005). We found a TRX reductase (*NTRA*; *At2g17420*) as one of the UPB1 direct target genes (Table S2). It was reported that *ntra* or *ntrb* single mutants do not show any phenotype (Reichheld et al., 2007) because of strong functional redundancy. However, the *ntra*/*ntrb* double mutant had a small meristem phenotype (Bashandy et al., 2010). NTR is important for reducing oxidized thioredoxin and thioredoxin plays an important role in providing reducing power to the peroxidases (Nordberg and Arner, 2001). These results also indicate that redox homeostasis in the root meristem plays an important role in the transition from cell proliferation to cell differentiation.

ROS Distribution Is Important for the Transition from Proliferation to Differentiation in Plants and Animals

ROS has also been shown to play an important role in maintaining the balance between cell proliferation and differentiation in animals. A redox-dependent signaling pathway controls the induction of cell division through the regulation of *cyclinD1* expression (Burch and Heintz, 2005). Distribution of specific ROS appears to act as an important signal at the transcriptional and posttranscriptional levels during cell-cycle progression in animal cells (Menon and Goswami, 2007). For example, in *Drosophila*, changing ROS levels can switch the status of hematopoietic cells from proliferation to differentiation (Owusu-Ansah and Banerjee, 2009). Moreover, it has been shown that manganese superoxide dismutase (MnSOD) activity regulates cell-cycle progression through modulation of ROS levels, which control expression of both the *cyclinB1* and *cyclinD1* genes in mouse cells (Sarsour et al., 2008). The authors proposed that $O_2^{\bullet -}$ regulates the proliferative cycle whereas H_2O_2 induces quiescence (Sarsour et al., 2008). This would be analogous to our model, in which $O_2^{\bullet -}$ accumulates in the meristematic zone and is necessary for proliferation, whereas H_2O_2 accumulates in the elongation zone when cells arrest division and begin differentiation. Intriguingly, we found that cell-cycle-related genes that are up-regulated by UPB1 including *cyclinB* and *cyclinD* genes do not appear to be direct targets according to our ChIP-chip data. Thus, it seems likely that UPB1 regulates cell-cycle progression indirectly by controlling ROS homeostasis.

Peroxidases are known to modify cell walls, mainly through lignin modification. In fact, class III peroxidases tend to localize in the extracellular space known as the apoplast (Passardi et al., 2006), where they can directly modify cell wall structure. Interestingly, we detected the UPB1 direct target, Per57-GFP fusion protein in the cytoplasm as well as in the apoplast (Figure 4C). According to our microarray data, in addition to peroxidases, UPB1 also regulates the "lignin synthesis" GO category. This suggests that UPB1 may act both directly and indirectly to modify cell walls. Thus, regulation of ROS status could act on the cell cycle to stop proliferation and, at the same time, act to modify cell walls to initiate cell expansion. The joint analysis of ROS-dependent changes to meristem cell number and cell length indicated that the ROS effects on cell division and cell length are correlated (Figure S5C). This supports a dual role for ROS, even though these functions would be compartmentalized as the cell wall modification would occur in the apoplast and the cell-cycle modulation would occur inside the cell.

UPB1 Regulation of Root Growth Is Independent of the Auxin/Cytokinin Signaling Pathway

It has been reported that auxin and cytokinin play an important role in controlling the balance between cell division and differentiation in the root meristem through two transcription factors, ARR1 and SHY2 (Dello Ioio et al., 2008). *UPB1* gene expression was not affected by either auxin or cytokinin, and in the *upb1-1* mutant, *SHY2* and *ARR1* expression levels and response to either auxin or cytokinin were similar to wild-type indicating that UPB1 acts through a pathway independent of this hormonal signaling pathway. It is surprising that two pathways can exert a powerful control on the balance between cell division and differentiation independently of each other. One explanation might be that the root has to integrate different types of information. Hormones usually serve as long distance signals, whereas the ROS pathway may play a key role in response to local cues and homeostasis. Interestingly, a role for ROS in local, rapid developmental decisions has emerged in animals. In zebrafish, a local gradient of ROS is used to rapidly trigger and execute a developmental program to recruit leukocytes to wounded tissue sites (Niethammer et al., 2009).

Although our results strongly suggest that UPB1 is involved in a pathway distinct from hormonal signaling for controlling the transition from proliferation to differentiation in the root, it remains possible that these two signaling systems converge at some level.

UPB1 May Act Non-Cell-Autonomously

There is an intriguing correlation between the height of the LRC and the point of transition from proliferation to differentiation. Comparison of transcriptional and translational fusions of *UPB1* suggests that UPB1 protein may be synthesized in the LRC and then move to the elongation zone where it becomes nuclear localized. Alternatively, UPB1 protein may be made at low levels in the elongation zone and have a long half-life, allowing it to accumulate in these cells.

Consistent with the first hypothesis, UPB1 is only 102 amino acids in length, suggesting that it could move passively through plasmodesmata. Additional support for this hypothesis came from analysis of a 3YFP-–tagged UPB1 protein, which is localized to the LRC in addition to all cells in the elongation zone. This altered localization pattern is presumably due to the larger size of the 3YFP tagged version, which may prevent passive diffusion from the LRC. The *35S::UPB1-3YFP* in *upb1-1* affected gene expression in the opposite manner to that of the *upb1-1* mutant. This finding indicates that the 3YFP tag does not disrupt UPB1 protein function as a transcriptional regulator. Taken together, these data suggest that at least some UPB1 is synthesized in the LRC and then moves to act in all cells of the elongation zone. In this way, UPB1 may act as a signal from the LRC indicating the proper location of the transition zone.

EXPERIMENTAL PROCEDURES

See Supplemental Information for details.

Plant Material and Treatment

Arabidopsis thaliana Columbia-0 (Col-0) was used as wild-type unless otherwise noted. The T-DNA insertion lines for *upb1-1* (SALK_115536) and *upb1-2* (SALK_133978) were confirmed using PCR with the primers listed in Table S3. For characterization of phenotypes, seeds were sown and allowed to germinate on vertically positioned media plates for 5 days, and then seedlings were transferred onto the treatment media.

Microarray Experiments

Total RNA was isolated from approximately 60 meristems and elongation zones of Col-0, *upb1-1*, and *35S::UPB1-3YFP #2* plants. Two biological replicates were performed for each experiment. Fragmented cRNA probes were prepared using the two-cycle amplification protocol recommended by Affymetrix. Samples were submitted to Expression Analysis Inc. (Durham, NC, USA) for hybridization to *Arabidopsis* whole genome ATH1 Affymetrix GeneChip.

ChIP-chip Experiment

pUPB1::UPB1-GFP lines were germinated and grown on the MS media for 6 days. Whole root tissue was fixed and chromatin immunoprecipitation (ChIP) was performed as described in Leibfried et al. (2005) with some modification, including the chromatin shearing by using a Bioruptor UCD-200 (Diagenode).

ROS-Related and Plant Hormone Treatments

Chemical treatments were performed by the transferring the 5-day seedling from MS media to the MS media containing those chemicals for 24 hr. Media contained H_2O_2 (100 μM), KI (1 mM), SHAM (100 μM), KCN (100 μM), DPI (100 μM), IAA (0.5 nM), and trans Zeatin (Zt) (5 μM), respectively.

Nitroblue tetrazolium (NBT) and dihydroethidium (DHE) were used for superoxide ($O_2^{\cdot-}$) staining and 3′-(p-hydroxyphenyl) fluorescein (HPF) and BES-H_2O_2-AC (WAKO, Japan) were used for hydrogen peroxide (H_2O_2) staining.

ACCESSION NUMBERS

All microarray data, including the tilling array design, were submitted to the NCBI GEO database under accession numbers GSE21876 and GSE21741.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at doi:10.1016/j.cell.2010.10.020.

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